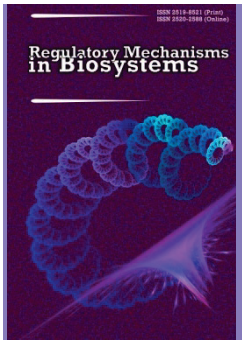

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.,
2022, 13(3), 308–316
doi: 10.15421/022240

Effects of transplanted mesenchymal stem cells on repair of the lung tissue of rats with experimental pulmonary fibrosis

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Article info

Received 01.07.2022
Received in revised form 25.07.2022
Accepted 26.07.2022

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Surtaieva, Y. V., Mazurkevich, A. Y., & Bokotko, R. R. (2022). Effects of transplanted mesenchymal stem cells on repair of the lung tissue of rats with experimental pulmonary fibrosis. Regulatory Mechanisms in Biosystems, 13(3), 308–316. doi:10.15421/022240

Pulmonary fibrosis is one of the commonest forms of interstitial lung diseases with poorly studied methods of its treatment in both human and veterinary medicines. Therefore, this paper focused on seeking alternative methods of its diagnostics and treatment. The article provides the results of the study of bronchoalveolar lavage fluid of rats with experimental lung fibrosis and influence of transplanted allogeneic mesenchymal stem cells of the bone marrow on stimulation of regenerative processes in damaged lung tissues. The studies were conducted on female Wistar rats with pulmonary fibrosis modeled using single transthoracic injection of solution of bleomycin hydrochloride. For the purpose of treatment, we used allogeneic mesenchymal stem cells introduced by various methods and the traditional treatment. We determined that best normalization of the parameters of the studied brochoalveolar lavage occurred in animals that received mesenchymal stem cells. The most active repair processes were in the experimental group that received the mesenchymal stem cells directly to the lung tissue. The animals that received intravenous injection of mesenchymal stemm cells were observed to have lower clinical parameters of the brochoalveolar lavage, but still better than such in the group treated traditionally. The lowest parameters were in animals that received the traditional treatment; they were greater than the phisological parameters, but significantly exceeded them in animals of the control group, indicating presence of inflammatory process in the lung tissue. The conducted cytological assays of the samples of the brochoalveolar lavage revealed that experimental animals with experimental pulmonary fibrosis had development of macrophage and lymphocytic reactions under the influence of transplanted mesenchymal stemm cells. We observed no atypical cells in all the experimental groups. This allows us to draw a conclusion that using stem cells by various methods of transplantation does not stimulate the onset of negative reactons (formation of atypical cells, metastatic processes, etc). Thus, the results of the study of the influence of transplanted mesenchymal stem cells demonstrate that in the conditions of experimental pulmonary fibrosis, the activity of regenerative processes in pathologically altered lung tissue may be an effective method of treatment of animals with this kind of pathology.

Keywords: bronchoalveolar lavage; pulmonary fibrosis; alogeneic mesenchymal stem cells; macrophages; regeneration; cellular therapy.

Introduction

The degree to which the etiopathogenesis of pulmonary fibrosis in animals has been studied, as well as diagnostic of it at this stage, requires more in-depth study using novel methods so as to achieve positive results in timely diagnosis of the disease and successful threatment of animals suffering this type of pathology.

Bronchoalveolar lavage of ill animals with a diagnostic purpose and laboratory assays of lavage fluid provide specialists with numerous parameters that are directly associated with the function, and therefore the structure of the lung tissue in physiological conditions, as well as in case of pathological process in the lungs. Bronchoalveolar lavage allows obtaining a biological sample that contains enough extravasated inflamatory cells and cytokines, which in turn reflect the microenvironment around the alveoli. Thus, assay may demonstrate progress or decline in studied pathology (Prasse et al., 2006; Yamaguchi et al., 2020). Samples of bronchoalveolar lavage fluid also contain secretion covering the apical surfaces of bronchial and alveolar epithelium. The amounts of aspirated fluid, cells and non-cellular components of bronchoalveolar lavage are determined by many factors. Therefore, to obtain informative material, it is important to correctly perform this procedure (Meyer et al., 2012). According to the data by various authors, the main components of the bronchoalveolar lavage of healthy animals are macrophages (90% of the overall number of cells). Concentration of neutrophiles is insignificant, and therefore the emergence of those cells is a sensitive indicator of presence of inflammatory process. Lymphocytes in bronchoalveolar lavage are usually absent in rats, mice and hamsters, but can account for up to 10% of cells of bronchoalveolar lavage. An indicator of inflammatory reaction based on the immune response is increase in the number of lymphocytes or eosinophiles in the samples of bronchoalveolar lavage (Henderson, 2005; Kodavanti, 2014).

Taking certain rinses from the respiratory tracts and lungs for the diagnostics of respiratory diseases or assays of toxicity of respiratory drugs currently require certain skills and attention on behalf of specialists. Using this method of gathering samples of the bronchoalveolar lavage of the respiratory system organs is popular because this method is economically simple to recreate and economicall beneficial (Matichin et al., 2019). In particular, bronchoalveolar lavage is usually used to evaluate various interstitial diseases of the lung tissue, which may provide some useful data (Raghu et al., 2018). Despite discussions regarding benefits of examining the cellular profile of bronchoalveolar lavage fluid in the diagnostics of fibrotic pulmonary diseases, the scientisits have numerously confirmed that this particular assay reflects the proliferative and fibrotic changes of the local environment of the lungs (Meloni et al., 2004; Vasakova et al., 2009). Bronchoalveolar lavage provides researchers with valuable and important data abount the mechanism behind the development of this pathological process in the lungs and may take a key role in the evaluation of the disease's severity (Paplińska-Goryca et al., 2019). Damages in alveolar epithelial cells and basal membrane were determined to cause internal alveolar proliferation of fibroblasts as a result of abnormal healing

(Selman & Pardo, 2001). Idiopathic pulmonary fibrosis is characterized by progressing deposition of fibrotic tissue in the lungs and decrease in the lungs' volume (Martinez et al., 2017). Treating patients suffering this form of the disease is not highly efficient. The average survival time is 2–3 years after the diagnosis (Prasse et al., 2006; Kim et al., 2015; Martinez et al., 2017).

Cellular therapy based on mesenchymal stem cells is a potential therapeutic approach for the treatment of various lung diseases (Bonfield & Caplan, 2010; Inamdar & Inamdar, 2013). Several studies revealed that mesenchymal stem cells are associated with decrease in damage to the tissues, inhibition of production of anti-inflammatory mediators, decrease in deposition of collagen in the extracellular matrix and in general promote regeneration of the tissues (Katsha et al., 2011; Toonkel et al., 2013). Moreover, mesenchymal stem cells produce paracrine factors with anti-inflammatory, anti-apoptosis and antifibrotic functions (Meirelles et al., 2009; Akram et al., 2013). Bone marrow-derived mesenchymal stem cells, both endogenous and exogenous stem cells, take part in the regenerative process (Rojas et al., 2005). Transfer of stem cells to damaged lungs is at least partially a result of the production of humoral mediators by damaged, but not normal lung, which are chemotaxic to stem stells (Rojas et al., 2005).

Mesenchymal stem cells of the bone marrow are a group of CD45-negative CD44H-positive cells, able to differentiate into various types of cells depending on the cultivation conditions, including endothelial, epithelial and neuronal cells, and also adipocytes (Lee et al., 2010). Mesenchymal stem cells are multipotent cells that are able to differentiate into a cellular lineage of mesodermal, endodermal and ectodermal cells (Weiss et al., 2008). Treating (regenerative) abilities of transplanted mesenchymal stem cells are based on their so-called houming capacity (Li, 2004), which is permeating to the bone marrow and attaching to specific regions in the bone marrow's microsurrounding, and also detect places of impairments in cellular homeostasis in the tissues in the organism, enter those areas and intensely reproduce by symmetric or asymmetric division, filling the intercellular space by daughter cells that obtain genotype of the local cells. In this particular way, in the physiological conditions, they restore the cellular composition of the organs in the organism throughout its life (Mazurkevych et al., 2013).

Studies indicate that transplanted mesenchymal stem cells of the bone marrow adopt best in the lungs, compared with other organs (Pereira et al., 1998; Krause et al., 2001), significantly relieve the inflammation and fibrosis in the lungs of mice having experimental fibrosis (Ortiz et al., 2003; Lee et al., 2010). Stem cell-based therapy could be used for the regeneration of the lungs and modulation of inflammatory and fibrotic processes (Toonkel et al., 2013). Other studies revealed that the processes of the regeneration likely involve several mechanisms of mesenchymal stem cells which lead to improvement of the results on model animals with pulmonary fibrosis (Toonkel et al., 2013; Tzouvelekis et al., 2013). It was pointed out that after introducing mesenchymal stem cells, they act as immune regulators that support cellular homeostasis, stimulating the endogenous mesenchymal stem cells and pulmonary precursor cells to self-regenerate (Yudhawati et al., 2020).

According to other studies, transplanted mesencymal stem cells migrate specifically to damaged alveolar epithelial cells and decrease the apoptosis of alveolar epithelial cells (Ortiz et al., 2003; Rojas et al., 2005), leading to differention of mesenchymal stem cells in alveolar epithelial cells (Zhao et al., 2008) through paracrine mechanisms (Zhen et al., 2008). Thus, transplantation of mesenchymal cells inhibits the production of pro-inflammatory mediators, decreases the thickness of alveoli in model of pulmonary fibrosis (Lan et al., 2015). Many reports revealed some advantages of using mesenchymal stem cells for mitigation of inflammatory reaction and fibrosis. However, therapeutic efficacy could be impacted by the number of transplanted cells, age of cells, time of transplantation and treatment period (Uji et al., 2013).

Model of bleomycin-induced fibrosis is the commonest pre-clinical in vivo model used to evaluate the potential therapeutic effect of mesenchymal stem cells in the conditions of pulmonary diseases (Cahill et al., 2016). In this model, early administration of mesenchymal stem cells during the initial phase of inflammation led to protective effect: decrease in the level of pro-inflammatory cytokines, decrease in the deposition of activated fibroblasts and collagen, and improvement in repair of epithelium (Ortiz et al., 2003; Moodley et al., 2009; Cahill et al., 2016).

The objective of this study was characterizing the changes in the cellular composition of rinsing fluid from the bronchoalveolar lavage in animals with experimental pulmonary fibrosis in the conditions of influence of transplanted alogenic mesenchymal stem cells of bone marrow so as to confirm its clinical benefits in this pathological process.

Materials and methods

The experimental animals were held in the conditions that meet the requirements of the Domestic Legislation, the Law of Ukraine "On Protection of Animals against Abuse" No. 3447-IV as of 21.02.2006, last amended on 08.04.2017, and Directive 2010/63/EU of the European Parliament and of the Council. In particular, the animals were kept in cages in 12 h daylight in the air temperature of 20–23 °C with free access to water and fodder in the conditions of the I. O. Povazhenko Surgery and Pathophysiology Department. The animals were allowed to be used in the studies according to the scheme received from the local commission of Bioethics of the National University of Bioresources and Nature Management of Ukraine (27.10.2020, Protocol No. 31-1).

The study objects were the samples of bronchoalveolar lavage fluid, obtained from female Wistar rats aged 4 months, with 277.0 ± 4.3 g live body weight and experimental pulmonary fibrosis caused by transthoracic administration of solution of hydrochloride bleomycin directly into the lung tissue. For 45 days, pulmonary fibrosis in animals was modeled using a single instillation of 0.3 mL of bleomycin solution into the lungs (Nippon Kayak Co., Ltd., Takasaki Plant, Japan), the active substance of bleomycin hydrochloride in the calculation of 1.0 mg/100 g of animals' body weight to 0.3 mL of 0.9% sterile physiological solution of sodium chloride, at the room temperature; the solution was singularly administered transthoracically directly into the lung tissue (Boiko et al., 2013). The place of administration of bleomycin hydrochloride was determined after palpation of the xiphoid process and imaginary dorsal line two thirds from the sternum. In that region, we made surgical field and carefully treated it with 70% alcohol solution, and carried out the puncture of the ribcage in the intercostal spaces using insulin syringe (BDMicroFinePlus, USA) with 31 G needle (0.25 x 6 mm) going full needle length and slowly injecting the solution directly into the lung tissue. This procedure was performed under a ligh anesthesia intramuscularly using 100 mg/mL Telazol (Zoetis, Spain) in the dose of 30 mg/kg (Plumb, 2008) and combination Medison 0.1% (Brovafarma, Ukraine) in the dose of 0.25 mg/kg intramuscularly (Plumb, 2008).

All animals with notable signs of pulmonary fibrosis on the 45th day (initial condition) were divided into 4 experimental groups, 20 individuals in each. The animals of the first experimental group received thransthoracic delivery of mesenchymal stem cells, directly into the lung tissue in the dose of 3 million cells per animal/individual singularly on the right side; animals of the second experimental group were injected with allogeneic mesenchymal stem cells intravenously in the same dose. Animals of the third gexperimental group were treated traditionally: injection of Dexametasone 4 mg/mL solution (KRKA, Slovenia) in the dose of 0.08 mg/kg intramuscularly for 3 weeks with 2-day interval, gradually reducing the dose, hyaluronidase 64 units solution (Lidaza-Biofarma, FZ Biofarma, Ukraine) in the dose of 0.85 units/kg intramuscularly for 3 weeks with 2-day interval (Boiko et al., 2013); and the control group received 0.3 mL of phosphate-buffer solution (Sigma, USA) transthoracically directly into the lung tissue on the right side.

The stem cells were obtained from the bone marrow of tibia, shoulder and femur of clinically healthy young animals (rats) by rinsing according to the methods developed by the Department's staff (Mazurkevych et al., 2014). The cells were cultivated in disposable Petri dishes containing DMEM – Dulbecco's Modified Eagle Medium (Sigma, USA) – 80%; FBS – Fetal Bovine Serum (Sigma, USA) – 20% and 10 μL/cm^3 – antimycotic antibiotic (Sigma, USA). The cultivation was carried out in CO_2 incubator in 37 ºC and 5% concentration of CO_2 until 90–100% formation of a monolayer. Attached cells were taken off using solution of 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma, USA) (Mazurkevych et al., 2014).

The first day of delivery of mesenchymal stem cells was considered day zero. Samples of blood and tissues for the laboratory assays were gathered on the 7th, 14th, 30th and 45th days of the experiment. The animals were withdrawn from the experiment by euthanasia through intra-abdominal injection of lethal dose of sodium Thiopenat (Thiopenat, Brovafarma – Ukraine), 40 mg/kg (Plumb, 2008), using insulin syringe (Medicare, Ukraine) with G 29 needle (0.33 x 13 mm). For the intra-abdominal injection, animal was held with its abdomen to the top and the head tilted below the hindlegs (Shoyaib et al., 2020). The abdominal cavity was visually divided into four parts, and injection was made into the right or left lower squares, avoiding the middle line. Needle was injected under the angle of 20–30°, performing the injection by pressing the plunger. After the procedure, we observed the animals until the end of their vital functions, particularly breathing and heartbeat. During the experiment, we saw no cases of death of animals in the experimental groups.

Then, we performed brochoalveolar lavage by open method by tracheal puncture after thoracotomy, spreading the wounds and further trachea visualization. Using insulin syringe (Medicare, Ukraine) with G 29 needle (0.33 x 13 mm), we carried out trachea puncture, and then four times washed it with 1.0 mL of 0.9% physiological solution of sodium chloride, and by slowly pulling up the syringe plunger, we carried out suction of the samples (Fig. 1). Total number of withdrawn fluid was 60–70% of the overall amount of administered fluid.

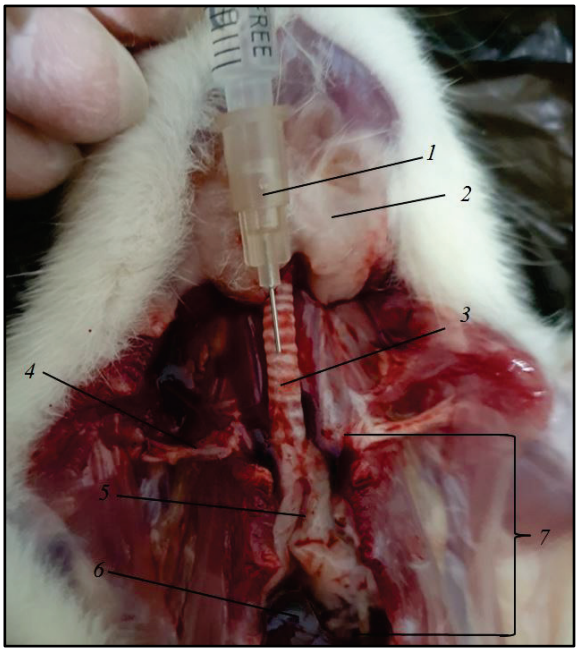

Fig. 1. Performance of bronchoalveolar lavage by open method: *1* – insulin syringe with needle, *2* – submandibular gland, *3* – trachea, *4* – clavicle, *5* – thymus, *6* – heart, *7* – thoracic cavity

For the microscopic (cytologic) analysis of the fluid taken using the bronchoalveolar lavage, it was put in centrifugation test tubes and centrifuged for 10 min at 3,000 rpm. Then, from the sediment, we prepared smears and stained them using Leukodif dyeing kit (Erba Lachema, Czech Republic), and counted the general amount of 100 cells under a microscope, noting the type of each cell (macrophage, neutrophile, eosinophile, lymphocyte, etc). In the general number of cells, which was 100 cells, we calculated the percentage of each type of cells.

The obtained results were statistically analyzed using Statistica 8.0 software pack (StatSoft, USA, 2012). The experimental data are presented as x ± SE (mean value ± standard error). The differences between the values of the control and experimental groups were determined using the Tukey test, where the differences were considered significant at $P < 0.05$.

Results

Changes in the cellular composition of the rinsing fluid from the bronchoalveolar lavage had its peculiarities and depended on the pattern of changes in the lung tissue, which in turn could be used for diagnostic purposes.

Microscopy of the smears of the bronchoalveolar lavage fluid revealed the following types of cells: alveolar macrophages, lymphocytes, neutrophiles, and – in insignificant amounts – eosinophiles, epithelial cells and erythrocytes. Furthermore, we performed studies to detect atypical cells and cellular detritus. Depending on the comparison advantage of one or the other type of cells over others, we distinguished the following types of bronchoalveolar lavage: with prevailance of relative amount of macrophages – macrophagous type, and such of lymphocytes – lymphocytic type. No cases with prevailance of eosinophiles or neutrophiles in bronchoalveolar lavage fluid were found.

The results of the examination of parameters in lavage fluid on day 7 of the experiment are given in Table 1. As we see from those results, the animals of all the experimental groups at this stage had significant increase in the number of macrophages and significant decrease in the number of lymphocytes, and also tendency toward increase in neutrophiles. In animals of experimental groups 1 and 2, single delivery of mesenchymal stem cells into the lung tissue and introvenuosly in the dose of 3 million cells per animal/animal promoted significant increase ($P < 0.01$) in the number of macrophages, indicating that in those conditions, the macrophagous properties of the lung tissues had been activated. At the same time, we also observed significant decrease in lymphocytes in the first and second experimental groups of animals ($P < 0.01$), which may obviously be related to the manifestation of immune-modulating effect of transplanted mesenchymal stem cells. Also, it is worth noting the dynamics of presence of lavage dendritic cells. Its decrease in animals of all the experimental groups – compared with the control – indicates weakening of the intensity of mortality of cellular elements of bronchoalveolar lavage. In animals of experimental group 3, which received the traditional treatment, we saw no significant cytologic changes compared with animals of the control group, indicating low level of regenerative processes.

Table 1

Cellular composition of bronchoalveolar lavage fluid of rats with experimental pulmonary fibrosis, the 7th day of the experiment (x ± SE, n = 5)

Parameter	Control	MSC into the lung tissue	MSC intravenously	Traditional treatment
Macrophages, %	57.6 ± 1.0^{a}	63.6 ± 1.5^{b}	65.2 ± 1.2^{b}	58.6 ± 0.7^{bc}
Lymphocytes, %	37.0 ± 0.7^{a}	27.0 ± 1.1^{b}	25.4 ± 1.2^{b}	33.2 ± 1.1^{bc}
Neutrophiles, %	8.6 ± 0.7^{a}	9.4 ± 0.9^{a}	11.4 ± 0.9^{a}	9.8 ± 0.7^{a}
Eosinophiles, %	0^{a}	0^{a}	0.2 ± 0.2^{a}	0^{a}

Note: different letters indicate significantly different values, one from another, within one line of the table according to the Tukey test ($P < 0.05$) taking into account Bonferroni correction; MSC – mesenchymal stem cells.

On the 14th day of the transplantation of alogeneic mesenchymal stem cells to the lung tissue of animals of the first experimental group, we observed significant increase ($P < 0.001$) in the number of alveolar macrophages, decrease in the number of lymphocytes ($P < 0.001$), and also manifestation of a small number of eosinophiles (Table 2). Increase in the number of macrophages and decrease in the number of lymphocytes indicate positive dynamics of repair of pathologically altered lung tissue. Emergence or increase in eosinophiles in blood is known to be associated with intensification of regenerative processes in the organism, as well as reaction to emergence of new immunogenic factors in the organism. Therefore, there are reasons to believe that mesenchymal stem cells transplanted intravenously have obviously stimualted hematopoiesis to a higher degree than after they had been injected intravenously. Also, it is worth noting the 2/3 decrease in cellular detritus, which may suggest higher activity of regenerative processes than in the other groups, and activity of reparatory processes and activity of detritus utilization that is associated with it. Animals of experimental group 3, which received the traditional treatment, remained the pattern of changes in the analyzed parameters.

On the 30th day of the experiment, animals of experimental groups 1 and 2, which had received delivery of mesenchymal stem cells into the lung tissue and intravenously, were observed to have significant 33.6% and 31.1% increases in alveolar macrophages, respectively ($P < 0.001$). In animals of the third experiment group, which received the traditional treatment, were seen to have significant decrease in macrophages compared

with animals of the control ($P < 0.05$), but it accounted for only 13.3%, indicating slower regeneration processes of the lung space (Table 3).

Table 2
Cellular composition of bronchoalveolar lavage fluid of rats with experimental pulmonary fibrosis, the 14th day of the experiment ($x \pm SE$, $n = 5$)

Parameter	Control	MSC into the lung tissue	MSC intravenously	Traditional treatment
Macrophages, %	56.6 ± 1.2^a	71.2 ± 2.0^b	74.4 ± 1.5^b	55.8 ± 2.0^a
Lymphocytes, %	41.4 ± 1.3^a	23.6 ± 1.1^b	17.2 ± 0.7^c	35.0 ± 1.0^a
Neutrophiles, %	5.6 ± 1.5^a	7.6 ± 0.9^a	7.6 ± 0.9^a	10.4 ± 1.6^a
Eosinophiles, %	0.4 ± 0.2^a	0.2 ± 0.2^a	0^a	0^a

Note: see Table 1.

Table 3
Cellular composition of bronchoalveolar lavage fluid of rats with experimental pulmonary fibrosis, the 30th day of the experiment ($x \pm SE$, $n = 5$)

Parameter	Control	MSC into the lung tissue	MSC intravenously	Traditional treatment
Macrophages, %	50.8 ± 1.5^a	76.6 ± 1.9^b	76.2 ± 1.7^b	58.6 ± 2.1^a
Lymphocytes, %	44.8 ± 1.7^a	16.6 ± 1.4^b	17.8 ± 1.5^b	31.0 ± 3.0^c
Neutrophiles, %	9.2 ± 1.1^a	6.0 ± 0.7^b	6.0 ± 0.7^b	6.8 ± 0.6^a
Eosinophiles, %	0.2 ± 0.2^a	0^a	0^a	0^a

Note: see Table 1.

The number of lymphocytes in samples of bronchoalveolar lavage at this stage significantly decreased in animals of all three experimental groups ($P < 0.001$), and the decrease pattern remained as well: 62.9% in animals of group 1 after delivering mesenchymal stem cells into the lung tissue, 60.4% in group 2 and 30.8% in group 3. In animals of experimental groups 1 and 2, after delivering the mesenchymal stem cells into the lung tissue and intravenously, we saw significant decrease ($P < 0.05$) in neutrophiles, and in animals of experimental group 3 – tendency toward decrease in their number compared with the control group, indicating lessening of the inflammatory process in the lung tissue under the effect of mesenchymal stem cells. Also, in animals of experimental group 2, there occurred 2/3 decrease in cellular detritus.

On the 45th day of the study, we observed significant increase ($P < 0.001$) in alveolar macrophages. In animals of experimental groups 1 and 2, which received mesenchymal stem cells, their number almost reached the physiological norm. In particular, we recorded significant 64.2% decrease in their number ($P < 0.001$) in animals of experimental group 1 and 62.6% decrease in animals of experimental group 2, compared with animals of the control group. Concentrations of lympocytes significantly decreased ($P < 0.001$) in groups 1 and 2. The number of neutrophiles in lavage fluid of animals of experimental group 1 and 2 dropped significantly, reaching the physiological values. The number of neutrophiles in animals of experimental group 3 (traditional treatment) remained at the level of day 30. In the third experimental group, where the animals had been treated traditionally, we saw no significant changes in smears of the bronchoalveolar lavage fluid on day 45 (Table 4).

Table 4
Cellular composition of bronchoalveolar lavage fluid of rats with experimental pulmonary fibrosis, the 45th day of the experiment ($x \pm SE$, $n = 5$)

Parameter	Control	MSC into the lung tissue	MSC intravenously	Traditional treatment
Macrophages, %	55.0 ± 2.0^a	85.6 ± 2.3^b	84.6 ± 1.2^b	60.4 ± 0.8^a
Lymphoytes, %	36.4 ± 3.7^a	13.0 ± 1.1^b	13.6 ± 1.6^b	32.6 ± 1.9^a
Neutrophiles, %	9.4 ± 1.1^a	3.8 ± 0.8^b	2.2 ± 0.7^b	8.0 ± 1.1^a
Eosinophiles, %	0.4 ± 0.2^a	0^a	0.2 ± 0.2^a	0^a

Note: see Table 1.

Throughout the period of the experiment, we observed a large amount of cellular material in smears from the rinsing fluid from the bronchoalveolar lavage, which confirms that we used the method correctly (Fig. 2). Also, in all the experimental groups of animals, we found no atypical cells. This leads us to think that neither delivery of stem cells into the lung tissue nor their intravenous injection stimulate the formation of atypical cells/metastatic processes. Throughout the experiment, we determined that in the lung tissue, after using the proposed methods of treatment, reparatory processes occurred with gradual decrease in lymphocytes and increase in the activity of alveolar macrophages, indicating high negative correlation (Fig. 3). During the traditional treatment, the activity of macrophages to lymphocytes had average negative correlation and was lower compared with the groups of animals that had received mesenchymal stem cell by different methods of delivery. After deliverig mesenchymal stem cells to the thoracic cavity of rats against the background of experimental pulmonary fibrosis, the cellular composition of bronchoalveolar lavage fluid was recovering at fast rates and was similar to intact animals, suggesting reparatory processes in the lung tissues.

Discussion

In this study, on the model of pulmonary fibrosis induced by hydrochloride bleomycin, we determined the efficacy of transplanted alogeneic mesenchymal stem cells, the activity of regenerative processes in experimentally damaged lung tissue of rats, and also confirmed the importance of using bronchoalveolar lavage for determining clinically significant processes in the lungs in the conditions of experimental fibrosis after 45 days of its development, when fibrotic changes in the lung tissue have already developed. The studies also confirmed the harmlessness (safety) of using alogeneic mesenchymal stem cells. Cellular-regenerative therapy for the treatment of lung diseases has been proposed earlier. The cells that have been tested the most are mesenchymal stromal cells of bone marrow (Lu & El-Hashash, 2019).

Since the airways contact the environment directly, they are especially vulnerable to unfavorable effects of inhaled pathogens and toxicants. Macrophages are important mechanism of host's immune protection from those pathogenic xenobiots. However, effective protection of the organism, wound healing and recovery of homeostasis require accurate control of the activity of macrophages. When operative mechanism of control are absent, macrophages re-activate, which causes exacerbation of acute damage to organ or development of chronic lung disease. This is complicated due to the fact that macrophages are not one homogenous cellular population, but rather subpopulations with unique phenotypical and functional abilities. Moreover, because macrophages are flexible cells, they are able to quickly change their phenotype (Laskin et al., 2019).

Developing new methods of treatment of lung diseases with fibrotic processes is important because of limited therapeutic possibilities of treatment methods as of now (Chen et al., 2018; Tzouvelekis et al., 2018; Harrell et al., 2019).

Treatment of induced pulmonary fibrosis in mice using mesenchymal stem cells demonstrates that mesenchymal stem cells can attenuate the severity of bleomycin-induced fibrosis, decrease deposition of collagen in the lungs, decrease the overall number of cells, number of neutrophiles in bronchoalveolar lavage fluid and increase the survival rate. The influence of various time of introduction of mesenchymal stem cells, which were obtained from the bone marrow, on the treatment of bleomycin-induced pulmonary fibrosis on model rats demonstrated that early (day 0) transplantation can prevent or decrease bleomycin-induced alveolar inflammation and pulmonary fibrosis, whereas late (day 14) transplantation can decrease alveolar inflammation. However, there were no evidences of improvement of condition of patients suffering pulmonary fibrosis (Zhang et al., 2019). Most results of the studies confirm that the treatment with mesenchymal stem cells can decrease bleomycin-induced inflammatory reaction in the lung tissue, decrease growth of inflammatory cells and expression of inflammatory cytocins, and decrease collagen deposition. Therefore, numerous pre-clinical trials have confirmed that mesenchymal stem cells have a great therapeutic effect on severe lung damage and pulmonary fibrosis (Zhao et al., 2021).

Bronchoalveolar lavage is a method used to evaluate the lung tissue, since it allows sampling the lower airways. In turn, differential number of cells of bronchoalveolar lavage is typical for certain lung diseases. However, some pathologies have distinctive features in the bronchoalveolar lavage, and need to be interpreted with other clinical and X-ray surveys. Development of novel methods of diagnostics would only increase the usefulness of bronchoalveolar lavage (Davidson et al., 2020). During pulmonary fibrosis, there occurs excessive deposition of extracellular matrix with following fibrosis, therefore there is an assumption about cross-

interaction between alveolar macrophages and fibrotic alveolar envrionement, and thus in modern pathogenesis of pulmonary fibrosis, stable inflammatory processes will cause initiation and spread of fibrotic reactions in the lung tissue (Zhang et al., 2018).

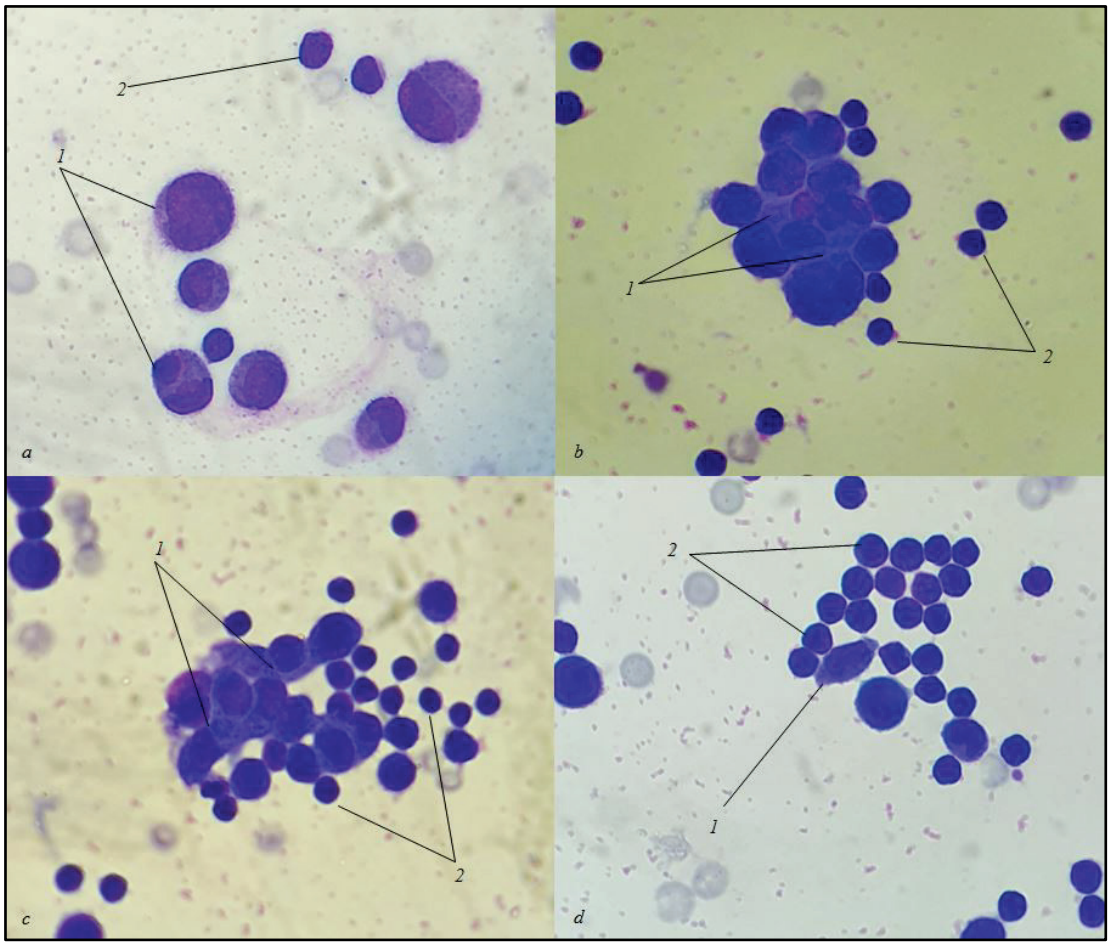

Fig. 2. Cells of bronchoalveolar lavage of rats on day 45 after injecting the mesenchymal stem cell into the lung tissue (*a*); mesenchymal stem cells intravenoiusly (*b*); traditional method of treatment (*c*); control (*d*): *1* – macrophages, *2* – lymphocytes (x100 lense, x10 ocular)

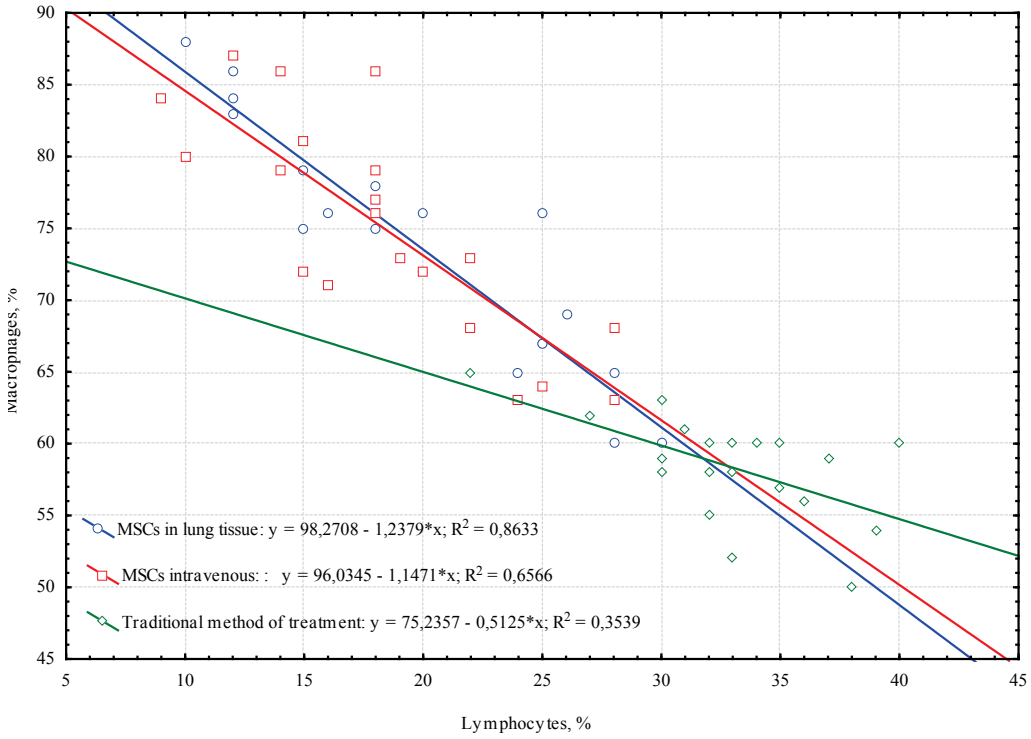

Fig. 3. Diagram of spread of macrophages depending on the number of lymphocytes in bronchoalveolar lavage, classified according to the treatment method throughout the experiment (n = 20)

Lung macrophages – depending on their locations – are divided into two groups: alveolar macrophages that are in alveoli (Guilliams et al., 2013), and interstitial macrophages that are in the parenchyma tissue (Bedoret et al., 2009). Macrophages mainly fuction in the host's system to remove pathogens by generating proinflammatory chemokines and cytokines, such as TNF-α, CCL2 and IL (Murray & Wynn, 2011; Saradna et al., 2018). Increase in the general amount of cells with mixed pattern, more often macrophages, dominating with <30% of total lymphocytes, varying increase in neutrophiles and insignificant increase in eosinophiles are characteristic for fibrotic pulmonary diseases (Veeraraghavan et al., 2003; Ohshimo et al., 2009; Meyer et al., 2012). However, heightened level of neutrophiles in bronchoalveolar lavage fluid is attributed to deterioration of the survival rate, though there is no unanimous opinion that those results of bronchoalveolar lavage may indicate the prognosis (Kinder et al., 2008).

Lung macrophages-1 and macrophages-2 are different subtypes of cells, and both take part in pathogenesis of pulmonary fibrosis. Macrophages-1 express high levels of proinflammatory cytokines, whereas macrophages-2 express high levels of cytokines. Because of different expression of cytokines, macrophages-1 and macrophages-2 play different roles in pathogenesis of pulmonary fibrosis. Macrophages-1 are typically responsible for wound healing once alveolar epithelium becomes damaged, while macrophages-2 are for processes of wound healing or stopping the inflammatory reactions in the lungs. Pulmonary fibrosis is a pathological consequence of altered wound healing in response to stable lung damage. Various regulatory cytokines, chemokines, mediators and immunoregulatory cells affect the polarization and chemostasis of lung macrophages. Those mediators interact and take effects on the longevity and severity of the disease through altered polarization of macrophage-1 and macrophage-2 cells. Thus, strategies oriented at modulation of phenotypes of lung macrophages can have a great potential for prophylaxis and treatment of pulmonary fibrosis in the clinical conditions. Fibrotic pulmonary disease is a lethal interstitial disease of lungs which is hard to diagnose and is poorly treated through the traditional therapy. Therefore, development of reliable and fast diagnostic methods and effective methods of treatment is very relevant, and aiming at activation and involvement of macrophages-2 could be vital therapeutic strategy (Zhang et al., 2018).

Mesenchymal stem cells are a population of non-hematopoietic multipotential stromal cells which can differentiate in the tissues that are derivates of a single germ layer. Mesenchymal stem cells are isolated from different areas, including fatty tissue, skeletal muscles, synovial sheath, spleen, thymus, lungs and amniotic fluid, but most often from the bone marrow. Mesenchymal stem cells have several functions, which make them promising therapeutic mean in the sphere of regenerative medicine, including secretion of anti-inflammatory cytokines and growth factor, migration of cells to the damaged places after delivery and ability to "save" cells by transfering functional mitochondria. In almost all medical articles on the subject, authors propose the possibility of autologic transplantation of cells, passing the immune rejection. Those properties, among other, make mesenchymal stem cells a promising therapeutic mean for the treatment of chronic pulmonary diseases with high morbidity and mortality, such as idiopathic pulmonary fibrosis, obstructive pulmonary diseases and obstructive bronchiolitis (Wecht & Rojas, 2016). By contrast, using alogeneic mesenchymal stem cells for the treatment of animals suffering those diseases does not lead to development of unsatisfactory immune reactions from the organism of recipient animal (Mazurkevych et al., 2014). Instead, transplanted alogeneic mesenchymal stem cells, because of their immunodepressive impact, provide tolerance of recipient animal's immune system cells. Another test of safety of transplanted alogeneic mesenchymal stem cells confirmed the results of previous studies.

Mechanisms through which mesenchymal stem cells decrease inflammations of the lungs and drive recovery of damaged organs have not been studied completely and, it seems, are related to several pathways, including contacts between cells, transfer of organelles and release of solved mediators or extracellular vesicules (Cruz et al., 2017).

The generally accepted hypothesis is that therapeutic action of mesenchymal stem cells depends on vitality of cells. However, this assumption was put into question, and the recent studies revealed thar dead or dying cells may improve the therapeutic effect, triggering immune cells of host (Luk et al., 2016; Galleu et al., 2017; de Witte et al., 2018; Weiss et al., 2019).

It was reported that mesenchymal stem cells display immunomodulating action through inhibition of proliferation of T-cells and secretion of anti-inflammatory cytokines and growth factors. Some reports revealed their ability to migrate from damaged place once introduced, and from there differentiate into certain types of cells so as to intitiate regeneration (Wecht et al., 2016). Mesenchymal stem cells have become a new potential therapeutic mean in treatment of pulmonary fibrosis, mainly because of their anti-inflammatory effect, migrating abilities and immune properties (Le Blanc et al., 2008). It is believed that mesenchymal stem cells initiate regeneration of epithelial tissue by providing endogenous stem cells to the damaged place and signaling about the local differentiation of stem cells (Wecht et al., 2016). Also, mesenchymal stem cells demonstrate anti-inflammatory and protective abilities by inhibiting inflammatory cytokines and producing growth fator, which can potentially promote the repair of the lung tissue and decrease in the inflammatory response (Huang et al., 2014).

In the lungs with chronic disease, mesenchymal stem cells promote recovery through several mechanisms: protection from microorganisms by secreting antibacterial peptides and increasing the phagocytic activity of monocytes (Alcayaga-Miranda et al., 2017); modulation of inflammation by stimulating the anti-inflammatory phenotype in alveolar macrophages (M2 cells) and T-regulatory cells, by decreasing the activity and proliferating anti-inflammatory cells (Prockop & Youn, 2012; Bernardo & Fibbe, 2013). It is interesting that mesenchymal stem cells always repair lung damage to a sufficient degree; some pre-clinical data revealed that mesenchymal stem cell can deteriorate pulmonary fibrosis (Srour & Thébaud, 2013).

Very important criterion in the sphere of cellular-regenerative therapy is the possibility of chromosomal mutations that may cause formation of tumors of cells transplanted to the lungs (Jeong et al., 2011). In our study, we observed no atypical cells in bronchoalveolar lavage fluid and macroscopic features of tumors during lung examinations. In this respect, other studies found no features of tumors in the lungs after day 21 (Alvarez-Palomo et al., 2020) and even after 12 months (Jun et al., 2011) after the transplantation of stem cells.

Scientists have conducted clinical studies evaluating benefits and safety of intravenous injection of mesenchymal stem cells obtained from the placenta for the treatment of idiopathic pulmonary fibrosis. Patients were injected 1×10^6 or 2×10^6 of mesenchymal stem cells per kg of body weight. The lungs function was monitored for 6 months. There were reports about insignificant side effects, but no signs of exacerbation of fibrosis were observed (Chambers et al., 2014).

The recent studies demonstrated that local (intratracheal) and systemic (intravenous or intraabdominal) influsion can be effective for decreasing the lung trauma, and therefore an optimal way of delivery is still unclear (Chimenti et al., 2012). As a variant, taking into account the advantages of local delivery aimed at the lung tissue, several studies revealed that stem cells need to be introduced in a lower dose (Chang et al., 2009; Chang et al., 2011). Intratracheal delivery of mesenchymal stem cells produced more effective regeneration of the airways compared with intravenous injection (Wong et al., 2007; Wong et al., 2009). Eighty percent of injected cells are found in the lungs several minutes after delivery. Mesenchymal stem cells are known to have tropism in relation to inflammated or traumatized places; and therefore, after delivery to experimental models of pulmonary diseases, they achieve lungs as first line barrier and are attracted by inflammatory microsurrounding (Leibacher et al., 2016).

So far, despite the increase in the number of clinical trials of the early phase using mesenchymal stem cells for the treatment of pulmonary diseases, no scientific consensus has been reached concerning the source of mesenchymal stem cells, strategies of dosing, therapeutic dose and production of cellular products (Rolandsson et al., 2020).

The obtained results demonstrate that single delivery of mesenchymal stem cells led to improvement in cellular composition, particularly macropages and disappearance of inflammatory process on the 45th day of the instillation of hydrochloride bleomycin.

To sum up, the results we obtained indicate decrease in inflammatory process, and improvement of macrophagous properties of the lung tissue.

Therefore, use of bronchoalveolar lavage is a significant method for diagnostics of fibrotic pulmonary diseases, particularly idiopathic pulmonary fibrosis.

Conclusion

Study of the effects stem cells take on the activity of regeneration of damaged lung tissue in the conditions of experimental pulmonary fibrosis on day 45 (in chronic period) is one of necessary directions of the modern veterinary cellular therapy. Therefore, after using mesenchymal stem cells in the conditions of experimental fibrosis of the lung tissue, we saw acceleration of the processes of lung tissue repair, indicated by the dynamics of parameters of cellular composition of bronchoalveolar lavage fluid. Depending on the method of delivering mesenchymal stem cells, we observed better effect after mesenchymal stem cells had been delivered directly into the lung tissue, when the studied parameters were close to the parameters of intact animals. Using the traditional method produced significantly lower results: the examined parameters were close to such of animals of the control group, and particualry, significantly high number of neutrophiles indicates presence of inflammatory process in the lung tissue.

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